Supporting Online Material for

Dynamic Regulation of Archaeal Proteasome Gate Opening As Studied by TROSY NMR

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Materials and methods

Protein expression

The *T. acidophilum* α and β proteasome subunits and the 11S activator from *Trypanosoma brucei* (PA26) were prepared as described previously (*S1, S2*), with mutations introduced using the Quikchange (Stratagene) approach. The monomeric version of the α subunit with the N-termini intact was generated by mutating six residues in the alpha-alpha interface to alanine (R57A/L58A/E60A/D84A/R86A/R130A). Methyl groups of Met were labeled as $^{13}\text{C}^1\text{H}$ by supplementing the D$_2$O-based growth media with 100 mg/L of $^{13}\text{C}^1\text{H}$ε labeled Met one hour prior to protein induction. PRE measurements were performed on samples labeled with either MTSL or TEMPO-Mal, as described below.

NMR Measurements

Methyl-TROSY ($^{15}\text{N}$-TROSY) spectra were recorded on per-deuterated protein samples dissolved in 100% D$_2$O (90% H$_2$O/10% D$_2$O), 25 mM potassium phosphate, pH 6.8, 50 mM NaCl, 1 mM EDTA, 0.03% NaN$_3$ at temperatures of 40 °C (α$_i$ or α$_i$+11S), 50 °C (α$_i$ or α$_i$α$_i$) or 70 °C (α$_i$β$_i$β$_i$α$_i$) using Varian 600 and 800 MHz spectrometers. NMR samples containing 11S were comprised of 100% D$_2$O buffer, 20 mM HEPES, pH 8.5, 0.03% NaN$_3$. Sample concentrations varied from 2 mM (monomer concentration) for measurement of exchange and ps-ns dynamics, 1 mM for amide PRE measurements, while spectra for Met methyl assignments and for quantifying methyl PRE values were
obtained on samples with protein concentrations of a few hundred µM. All data were processed and analyzed with the NMRPipe suite of programs (S3) or the program Sparky (S4). PRE values were obtained by recording a series of TROSY-based \(^1\text{H}-^{13}\text{C}\) or \(^1\text{H}-^{15}\text{N}\) data sets where a delay for quantifying the \(^1\text{H}\) relaxation rate was varied parametrically (S5). These PRE values were subsequently used as restraints in simulated annealing restrained molecular dynamics calculations of ensembles of structures of the gating residues, as discussed in detail below. Pulse program code for experiments is available upon request.

**MTSL spin labeling**

Labeling of \(\alpha\)-subunits with spin-label was achieved by introducing a single Cys mutant at the desired position (R20, D72, S95, V107, R115, Q143). Purified Cys mutants, stored with 5 mM DTT, were buffered exchanged into 1 mL of degassed 50 mM KPi, pH 7.4, 1 mM EDTA, 0.02% NaN\(_3\) buffer. MTSL spin label (Toronto Research Chemicals) was subsequently added in 3-fold molar excess and the reaction allowed to occur for 1 h at room temperature. The reaction was terminated by exchange into NMR buffer. All samples were submitted for mass confirmation (mass spectrometry) to ensure proper labeling. No MTSL labeling of the intrinsic C151 was observed under these conditions. In case of the 11S activator, the intrinsic Cys residues at positions 78 and 83 were mutated to Ser prior to introduction of Cys at position 108. Note that the spin-label is attached to all 7 equivalent positions (one per monomer) in \(\alpha_7\) or in the 11S activator.
After NMR datasets were recorded on the oxidized samples (see below), reduced samples were generated through the addition of excess DTT, followed by buffer exchange.

**TEMPO spin labeling**

The MTSL labeling approach described above failed when label was attached to the mobile N-terminal gating residues (used for recording amide $^{1}H^{N}$ PRE values). Mass spectrometry analysis of MTSL-labeled samples used to record amide $^{1}H^{N}$ R$_2$ experiments established partial loss of spin-label for the G4C mutant, likely due to a thiol-disulfide exchange reaction between the flexible N-termini that is very effective at elevated temperatures (50°C). Therefore, a TEMPO-Mal label (Toronto Research Chemicals) was employed that cannot exchange. Samples were prepared as described for MTSL labeling with the exception that an excess of 1.5 fold label was used that was allowed to react overnight. This label cannot be reduced; relaxation measurements were recorded on labeled and unlabeled samples.

**Data Analysis:**

**(A) Magnetization Exchange**

Two-dimensional magnetization exchange experiments (S6) were recorded to quantify exchange between states A, B and C. The pulse sequence code is available upon request. Briefly, $^{1}H$ magnetization is recorded during an evolution delay, preceding a mixing period during which magnetization exchange is allowed to occur. The experiment is constructed in a manner such that only $^{1}H$ magnetization one-bond coupled to $^{13}C$ is selected (*i.e.*, only Met $^{1}H^{\alpha}$ magnetization) with the magnetization of interest immediately
prior to the mixing period of the form, $I_z C_z$ (longitudinal order), where $I_z$, $C_z$ denote proton and carbon $Z$-magnetization, respectively. During the mixing period longitudinal order is allowed to exchange, so that magnetization in state A can transfer to states B or C or remain in state A, for example. Subsequently, after the exchange period, anti-phase $^1$H magnetization is refocused with respect to the one-bond $^1$H-$^{13}\text{C}$ scalar coupling and $^1$H signal is recorded. This gives rise to a two-dimensional exchange spectrum with diagonal peaks at $(\omega_j, \omega_j)$ ($j \in \{A,B,C\}$) corresponding to magnetization that does not exchange during the course of the experiment and cross-peaks at $(\omega_m, \omega_n)$ ($m \neq n$, $m,n \in \{A,B,C\}$) that arise from exchange between states $m$ and $n$. The equations governing the magnetization exchange process are given by:

\[
\frac{d}{dt} \begin{bmatrix} M_A(t) \\ M_B(t) \\ M_C(t) \end{bmatrix} = \begin{bmatrix} -k_{AB} - k_{AC} - R_A & k_{BA} & k_{CA} \\ k_{AB} & -k_{BA} - k_{BC} - R_B & k_{CB} \\ k_{AC} & k_{BC} & -k_{CA} - k_{CB} - R_C \end{bmatrix} \begin{bmatrix} M_A(t) \\ M_B(t) \\ M_C(t) \end{bmatrix}
\]

where $M_n(t)$ is the magnetization of state $n$ at time $t$, $k_{mn}$ is the rate constant for exchange from state $m$ to $n$ and $R_n$ is the decay of longitudinal order in state $n$. Assuming that the magnetization reports faithfully on the equilibrium populations of each state, the solution to the above equation is:

\[
M_{m \rightarrow n}(t) = \lambda \vec{v}_n \exp(\mathbf{K}t) \tilde{p}_m
\]

where $\vec{v}_n = \{[1 0 0], [0 1 0], [0 0 1]\}$ and $\tilde{p}_n = \{ p_A, 0, 0 \}

\begin{bmatrix} 0 \\ p_B \\ 0 \end{bmatrix}$ for $n=\{A,B,C\}$, $p_A, p_B, p_C$ are the fractional populations of each state, $\mathbf{K}$ is the 3X3 exchange matrix in Eq. [1] above and $\lambda$ is a fitting parameter corresponding to the total
magnetization at a mixing time of 0. Values of \(p_A, p_B\) and \(p_C\) were measured from a \(^1\text{H}-^{13}\text{C}\) HMQC spectrum recorded with a long relaxation delay (3 s) to allow for equilibration of initial magnetization. Auto- and cross-peak time profiles (comprising delays of 0.05, 0.1, 0.2, 0.3, 0.4 (twice), 0.5 (twice) 0.6, 0.7, 0.8, 0.9, 1.0 s) were fitted simultaneously to Eq. [2] involving 7 adjustable parameters that included three exchange rate constants \((k_{\text{EX(AB)}} = k_{AB} + k_{BA}), k_{\text{EX(AC)}} = k_{AC} + k_{CA}, k_{\text{EX(BC)}} = k_{BC} + k_{CB})\), three \(R_n\) rates \((R_A, R_B, R_C)\) and \(\lambda\). Errors in the extracted exchange rates were obtained using a jack-knife simulation approach where 30 data sets were created each with 40% of the time points removed \((S7)\). The standard deviations in parameters from the resultant 30 fits are reported as the errors. The errors on populations extracted from HMQC spectra were estimated at 2% based on repeat measurements.

(B) Extraction of \(S^2\) values reporting on the amplitudes of ps-ns time-scale motions

\(S^2\) axis values for the Met C\(^e\)-S\(^\delta\) bond vector were measured and analyzed as described by Tugarinov et al. \((S8)\) using an approach which measures the time dependencies of sums \((I_a)\) and differences \((I_b)\) of magnetization derived from methyl \(^1\text{H}\) single-quantum transitions. Values of \(I_a\) and \(I_b\) were measured for time points of 1.5, 2, 3, 4, 5, 6, 7, 8, 10, 12, 14, 18, 22, 28, 34, 40, 50, 60 ms. The profiles \(I_a/I_b\) were fitted to:

\[
I_a/I_b = \frac{-0.5 \eta \tanh(\sqrt{\eta^2 + \delta^2 T})}{\sqrt{\eta^2 + \delta^2} - \delta \tanh(\sqrt{\eta^2 + \delta^2 T})}
\]  

[3.1]
where \( \delta \) is a parameter that is related to the \(^1\)H spin density around the methyl group in question and:

\[
\eta \approx \frac{9}{10} \left[ P_2 \left( \cos \theta_{\text{axis,HH}} \right) \right]^2 \frac{S^2 \gamma^2 \hbar^2 \tau_c}{\gamma_H^6 \tau_H^6} \tag{3.2}
\]

with \( \tau_c \) the tumbling time of the assumed isotropically rotating particle, \( \gamma_H \) is the gyromagnetic ratio of a proton spin, \( r_{\text{HH}} \) is the distance between pairs of methyl protons (1.813 Å), \( P_2(x) = \frac{1}{2} (3x^2 - 1) \), and \( \theta_{\text{axis,HH}} \) (90°) is the angle between the methyl three-fold axis and a vector that connects a pair of methyl \(^1\)H nuclei. A value of \( \tau_c = 60 \) ns has been used in the present analysis, \( \frac{1}{2} \) the value of the \( \alpha, \alpha \) particle (SI). Values of \( S^2 \) are listed in Fig. 3A; errors in \( I_a, I_b \) values were estimated from the noise floor of spectra and fitted errors in \( S^2 \) obtained via the covariance matrix method (S7) were always less than 0.01.

(C) Measurement of PRE values

MTSL spin labels were placed at positions R20C, D72C, S95C, V107C, R115C, Q143C in \( \alpha \), or at position A108C in the 11S activator and Met methyl \(^1\)H relaxation rates were recorded in the oxidized (\( R_{\text{ox}}^2 \)) and reduced (\( R_{\text{red}}^2 \)) states to obtain the methyl \(^1\)H PRE rate, \( \Gamma_{2,\text{CH3}} = R_{\text{ox}}^2 - R_{\text{red}}^2 \). Relaxation rates were measured using a \(^1\)H-\(^{13}\)C HMQC-type pulse scheme (available upon request) that selects for only the slowly relaxing magnetization components, with a variable delay included during which transverse \(^1\)H magnetization is allowed to relax (S8). This delay was set to 1, 5, 10 (twice), 15, 20, 30, 50, 80 ms. Measured peak intensities in two-dimensional correlation maps were fitted to extract \( R_2 \) as described in the legend to Fig. S5, with errors in \( R_2 \) estimated from the
covariance matrix method (S7). Correlations disappeared for some residues in the oxidized state; in these cases a lower bound for $R_2^{ox}$ was estimated from the relation

$$\frac{I_{ax}}{I_{red}} = \frac{\exp(-2R_2^{ox}\tau)}{\exp(-2R_2^{red}\tau)} \left( \frac{R_2^{red}}{R_2^{ox}} \right)^2$$

where $I_{red}$ is the intensity of the correlation in the $^1$H-$^{13}$C spectrum recorded of the protein with spin label in the reduced state while $I_{ox}$ is set to 3 times the noise floor. Only contributions to relaxation of the methyl $^1$H probe (i.e., not the methyl $^{13}$C) are considered. The value $\tau$ is the time for transfer of magnetization between $^1$H and $^{13}$C spins and is set to 3.5 ms. No significant changes in $R_2$ rates were obtained from back-to-back measurements (differences were within experimental errors) and mass-spectrometric analysis showed that samples with the MTSL spin-label remained attached to the protein after each experiment.

A TEMPO-Mal spin-label was attached to G4C in $\alpha_7$ and amide $^1$H$^N$ $R_2$ values obtained from a series of 2D $^1$H-$^{15}$N TROSY-based HSQC data sets that included a variable delay during which $^1$H transverse relaxation was allowed to occur (pulse sequence available upon request). Relaxation rates were calculated from delay values of 6.5 (twice), 8, 10, 13, 16 ms, with errors estimated on the basis of the covariance matrix approach (S7). For those residues whose correlations were bleached out in the oxidized state a lower bound for $R_2^{ox}$ was obtained from:

$$\frac{I_{ax}}{I_{red}} = \exp(-(R_2^{ox} - R_2^{red})\tau) . \frac{R_2^{red}}{R_2^{ox}} \cdot \frac{\exp(-2R_2^{ox}\tau) + \exp(-R_2^{ox}\tau)}{\exp(-2R_2^{red}\tau) + \exp(-R_2^{red}\tau)}$$

[5]
where $\tau = 4.6$ ms and $I_{ox}$ is set to 3 times the noise floor. Sample integrity throughout the experiment was ensured via duplicate experiments (differences in rates were within measurement errors) and by mass spectrometric analysis on samples after data sets were recorded.

**(D) Structure calculations**

Site-specific $^1$H$^N$ and methyl $^1$H $\Gamma_2$ rates were converted to distances from the proton in question to the paramagnetic centre using the formula (S9):

$$r = \left[ \frac{\beta}{\Gamma_2} \left( 4\tau_c + \frac{3\tau_c}{1 + \omega_H^2 \tau_c^2} \right) \right]^{1/6} \quad [6]$$

where $\beta = 1.23 \times 10^{-44} \text{ m}^6 \text{ s}^{-2}$, $\omega_H = 2\pi 600 \times 10^6 \text{ rad} \text{ s}^{-1}$ and $\tau_c = 60 \text{ ns}$ for $\alpha_r$. Distance values obtained in this manner must be considered as upper-bounds because Eq. [6] does not include the effects of dynamics that are likely considerable in the present system. Thus, the upper bounds for distance restraints used in all calculations were set as the distances reported by Eq. [6] + 2 Å (unless the error in $r$ was larger than 2 Å), with the lower bound set to 1.8 Å.

As described in the text three separate peaks were observed for M-1 and M1 corresponding to states A, B and C, that arise from gating termini that are outside (A) or inside (B,C) the antechamber. On the basis of measured PRE rates the largest distance difference between corresponding probes in states B and C is 3 Å (20 vs 23 Å), quantified for the spin label at position 72 and for Met1 protons. This difference is relatively small in relation to the absolute distance. Further because the majority of the calculated distances between any of the paramagnets used and the methyl protons of M1 in states B
or C are within error (with the same situation holding for states B and C of M-1), states B and C were not distinguished in structure calculations, but rather were considered to be part of a single ‘in’ conformation. Thus, two classes of restraints were considered for the Met methyl groups at positions -1, 1 and 6 (‘in’ derived from PRE values associated with Met groups of states B/C and ‘out’ from PREs measured from peaks associated with Met in state A). Computations have established that for slow exchange between ‘in’ and ‘out’ with life-times on the order of several seconds (see Fig. 3C) there is no ‘contamination’ between PRE values measured for each of the ‘in’ and ‘out’ conformations.

The number of termini in each of the ‘in’ and ‘out’ states was estimated from peak volumes in fully relaxed HMQC data sets, with close to 2 and 3 termini in the ‘in’ position for WT and Y8G/D9G \(\alpha_7\), respectively. However, no information is obtained from NMR experiments regarding which of the 7 termini are inside and outside of the antechamber – structures were therefore calculated for the different possibilities (see below). There are \(\frac{7!}{7k!(7-k)!}\) ways in which \(k\) termini located in the ‘in’ position can be distributed around the \(\alpha_7\) heptamer, taking into account the equivalence of circular permutations. For example, there is only one way of placing one chain inside the lumen of the antechamber, but three different possibilities of having two chains inside the antechamber – when they are next to each other (denoted by “[1 1 0 0 0 0 0]” where 1 and 0 indicate ‘in’ and ‘out’), separated by one (“[1 0 1 0 0 0 0]”) or two (“[1 0 0 1 0 0 0]”) chains that are outside the antechamber. From volume intensities of peaks in HMQC spectra 24% and 45% of the N-termini are inside of the lumen for WT and Y8G/D9G \(\alpha_7\), Fig. 2C. Although there are many ways of distributing 7 termini between ‘in’ and ‘out’ to
achieve 24% and 45% ‘in’, one simple way is that WT $\alpha_7$ has 32% 1 chain ‘in’, 68% 2 chains ‘in’, while Y8G/D9G $\alpha_7$ is comprised of 15% 4 chains ‘in’, 85% 3 chains ‘in’. Therefore, structures were calculated for all possible permutations from 1 to 4 chains within the antechamber (14 possibilities, as shown below – 1 ‘in’: [1 0 0 0 0 0 0]; 2 ‘in’: [1 1 0 0 0 0 0], [1 0 1 0 0 0 0], [1 0 0 1 0 0 0]; 3 ‘in’: [1 1 1 0 0 0 0], [1 1 0 1 0 0 0], [1 1 0 0 1 0 0], [1 0 0 1 0 0]; 3 ‘in’: [1 1 1 0 0 0 0], [1 1 0 1 0 0 0], [1 1 0 0 1 0 0], [1 0 1 0 0 1 0], [1 0 1 0 0 0]; 4 ‘in’: [1 1 1 0 0 0 0], [1 1 0 1 0 0 0], [1 1 0 0 1 0 0], [1 1 0 0 0 1 0], [1 1 0 0 1 0 0], [1 1 0 1 0 0]). At the beginning of every structure calculation each N-terminus was assigned restraints corresponding to either the ‘out’ (A) or ‘in’ (B/C) states consistent with one of the 14 permutations indicated above.

Permutations of termini ‘in’ and ‘out’, as described above, where black and white correspond to ‘in’ and ‘out’, respectively.

The proteosome crystal structure determined by Lowe et al. (S10) (1PMA) was the starting point for structure calculations. The first 12 N-terminal residues are not visible in 1PMA but coordinates for residues 7-12 are available from the structure of the 20S CP-11S activator complex (S11) (1YA7); these were ‘grafted’ onto 1PMA. Backbone atoms of the Cys residue and the added spin-label (either MTSL or TEMPO-Mal) were superimposed with the backbone atoms at the appropriate site in each $\alpha$-
subunit. The molecule with added spin labels and all missing protons was minimized prior to structure calculations, with all torsion angles for amino acids up to and including position 6 (-3–6; i.e., those for which no structural information is available) randomized. Although all of the experimental PRE measurements were performed on samples with a paramagnet attached to only one site per α-subunit (for example position 95), corresponding to 7 paramagnets per α7, calculations were performed with all of the paramagnets in place (i.e., at positions 4, 20, 72, 95, 107, 115, 143) because the restraints that are measured in experiments and used in calculations are the distances from the proton in question to the oxygen atom of the nitroxide. Since all of the restraints are to be included in each calculation (i.e., for a given molecule), the simplest approach is to include all spin-labels in the molecule for the purposes of calculation, but to ensure that the spin-labels do not ‘see’ each other (see below), following the lead of Clore and coworkers (S12).

During the structure calculation residues 14-233 of each α-subunit within α7 were fixed to their positions in the 1PMA crystal structure, while residues -3–13, the side-chain atoms of the Cys coupled to the spin labels and the spin-label atoms were allowed to move (see below). The attached spin-labels were prevented from clashing with backbone and Cβ,Hβ atoms of α7, but were allowed to overlap with other side-chain proteasome positions to reflect the fact that while side-chains were fixed during the computation they would normally be dynamic and thus would be able to move to accommodate the label. In a similar manner, spin labels from distinct positions (i.e. R115 or S95) were allowed to overlap with each other during the structure calculation since
experiments were performed on samples with spin label attached to only a single position, as described above.

Structures were calculated using the XPLOR-NIH 2.23 software (S13) package by modification of a standard sample script, eginput/gb1_rdc/anneal.py, that is available for downloading from the group of Marius Clore (http://nmr.cit.nih.gov/xplor-nih/). All PRE distance restraints, including Met methyl $^1$H restraints derived from label at positions 20, 72, 95, 107, 115, 143 and amide $^1$H restraints from the nitroxide at residue 4 were included in the calculations. PRE restraints were implemented using standard NOE potentials in XPLOR (S14, S15) with values of $r$ computed as:

$$r_{CH_i} = \left( \sum_{\text{LABEL}} r_{\text{LABEL}}^6 \right)^{-1/6}$$
$$r_{NH} = \left( \frac{1}{7} \sum_{N_{\text{LABEL}}} \sum_{NH_{\text{LABEL}}} r_{NH,\text{LABEL}}^6 \right)^{-1/6}$$

[7]

During the first five steps of the structure calculation protocol (see below) separate methyl restraints were implemented for each N-terminus (there was no separate averaging over the $k$ ‘out’ termini or over the $7-k$ ‘in’ termini and hence no prefactor in front of the summation for $r_{CH_i}$ of Eq. [7], see below). This was necessary to ‘force’ the correct number of termini ‘in’ and ‘out’ in the initial stages of the refinement so that when the [1 1 0 0 0 0 0] structure is calculated, for example, 2 (and only 2) adjacent termini are constrained to be ‘in’, while 3 termini are inside for the [1 1 1 0 0 0 0] structure. Note that all PRE constraints could be satisfied with just a single terminus within the lumen, although it is clear from peak intensities in HMQC spectra that on average approximately 2 (WT) or 3 (Y8G/D9G) termini are inside. In later stages of the
calculation, when the correct number of termini are positioned within the lumen, an
ensemble averaging procedure was implemented for calculation of N-terminal methyl-
nitroxide distances, as described below.

Briefly, the following steps were performed (script available upon request):

1. **High temperature torsion angle (TA) dynamics** (2000 steps @ 3500 K) to remove
   steric clashes between N-termini and $\alpha_7$ residues created by randomization of torsion
   angles for residues -3–6 (see above). The following energy terms were employed: BOND
   (1 kcal mol$^{-1}$ Å$^{-2}$), ANGLE (0.4 kcal mol$^{-1}$ rad$^2$), IMPROPER (0.1 kcal mol$^{-1}$ rad$^2$), NOE
   (distances derived from PRE measurements) (0.2 kcal mol$^{-1}$ Å$^{-2}$), RAMA (0.002 kcal mol
   $^{-1}$ rad$^2$) and VDW (10 kcal mol$^{-1}$ Å$^{-2}$ between N-termini (residues -3-13) and the rest of
   $\alpha_7$; 0 kcal mol$^{-1}$ Å$^{-2}$ between the N-termini themselves). Disabling the interactions
   between the N-termini increased significantly the convergence rate of the structure
   calculations. These values were maintained until step 6.

2. **High temperature TA dynamics** (2000 steps @ 3500 K) with PRE distance restraints
   increased to 2 kcal mol$^{-1}$ Å$^{-2}$ to generate structures with the correct numbers of N-termini
   in the ‘in’ and ‘out’ positions. (Recall that separate structure calculations are performed
   for different numbers of termini ‘in’ and ‘out”).

3. **High temperature TA dynamics** (1000 steps @ 3500 K) with the VDW energy term
   turned on also for residues -3–13 (0.1 kcal mol$^{-1}$ Å$^{-2}$), prior to additional increases in
   VDW energies between N-termini in the subsequent step.

Note: In steps 1-3 the positions of the spin-labels are fixed to increase the convergence
rate of the calculations.
4. **TA annealing**: temperature ramped down from 3500 K to 25 K in steps of 200 K, with 2 ns of dynamics at each step. ANGLE (0.4 to 1.0 kcal mol\(^{-1}\) rad\(^{-2}\)), IMPROPER (0.1 to 1.0 kcal mol\(^{-1}\) rad\(^{-2}\)), VDW (0.1 to 4.0 kcal mol\(^{-1}\) Å\(^{-2}\)), RAMA (0.002 to 1.0 kcal mol\(^{-1}\) rad\(^{-2}\)) and NOE (2 to 300 kcal mol\(^{-1}\) Å\(^{-2}\)) energy terms are ramped up multiplicatively at this stage. Spin-labels are allowed to move.

5. **TA minimization**: 500 steps.

6. **Cartesian annealing**: temperature ramped down from 650 K to 25 K in steps of 100 K with 1 ns dynamics at each step. At this stage ‘ensemble-averaging’ was introduced for the N-terminal methyl group restraints. Briefly, distances involving methyl protons of M-1, M1 and M6, attached to highly dynamic termini, were computed separately for each class of ‘in’ and ‘out’ termini. In the case where \(k\) termini are in the ‘out’ position, with \(7-k\) in the ‘in’ state, values of \(r\) were calculated according to:

\[
r_{in} = \left( \frac{1}{7-k} \sum_{CH} \sum_{LABEL} r_{CH,LABEL}^{-6} \right)^{-1/6} \quad r_{out} = \left( \frac{1}{k} \sum_{CH} \sum_{LABEL} r_{CH,LABEL}^{-6} \right)^{-1/6}
\]  

[8]

Eq. [8] is derived under the assumption that chains within a group (i.e., within the ‘in’ or ‘out’ group) exchange with each other rapidly, while the interconversion between ‘in’ and ‘out’ conformations is slow (on the order of several seconds, Fig. 3 of the text). There are several lines of evidence that argue in favor of rapid exchange between termini within a given conformation (i.e., rapid exchange between ‘in’ termini). First, \(S^2\) values for M-1, M1 and M6 are < 0.1, much lower than expected for a Met residue with ‘typical’ dynamics \((S16)\). Second, amide correlation spectra recorded on WT \(\alpha_7\) with spin-label
attached to position -2, 1 or 4 show levels of peak attenuation that are only consistent with rapid exchange of termini that exposes all 7 amide sites within the anti-chamber to spin-label. Simulations that we have performed show that if the 2 ‘in’ termini (with attached spin-labels) were sequestered to separate single sites then amide probes in helices 1 and 2 would be attenuated by no more than 45%. However, attenuation levels of over 60% are observed for 8 amide peaks from residues inside of the chamber (4 peaks over 70%; peaks for V87 and F91 completely disappear), consistent with a highly disordered N-terminus. Third, MTSL spin-label attached to positions -2, 1 and 4 was partially lost during experiment (hence TEMPO-Mal was used, see above), very likely reflecting a disulfide interchange reaction that is predicated on flexibility of the N-termini. Finally, it is worth emphasizing that the differences in Eqs. [7] and [8] reflect the fact that amide restraints involve NH protons that are all attached to the barrel structure (amides of the gating residues are all broadened so that no restraints are obtained for them, Fig. S1); by contrast, different restraints are associated with methyl groups of M-1, M1 and M6 depending on whether they are attached to N-termini in the ‘in’ or ‘out’ positions.

7. Cartesian minimization for 500 steps.

Five hundred structures were calculated for each of WT and Y8G/D9G α; with 360 satisfying all distance restraints (violations less than 0.5 Å). Additional structures were obtained by an ensemble refinement with a pair of α; structures refined simultaneously,
however, no improvement was noted, not surprisingly since all distances were already satisfied in the single-structure calculations. It is worth noting that there is an element of ensemble refinement inherent to the single structure calculations since each involves 7 termini as well as 7 spin labels of a given type, with averaging over labels and probes during the calculations. Structural statistics are reported for WT and Y8G/D9G α₇ below, based on the 10 lowest energy structures for each possible permutation comprising 2 (WT) and 3 (Y8G/D9G) termini ‘in’ (3 and 5 distinct ways of placing 2 and 3 termini in, see figure above).

A set of structures calculated using the above protocol is shown in Figure 2D. It is worth noting that, as expected, the proteasome cannot cleave the ‘in’ state gating residues; from the ensemble of ‘in’ configurations the average distance from the α-annulus to M1 Cα is 16 ± 3 Å, with the N-terminal residue in our construct, Gly-3, no more than 32 Å from the annulus, placing it well outside of the active sites in the intact proteasome (Fig. 1A).

(E) Calculation of accessible surface area of the α-annulus

A property of interest is the accessible surface area of the α-annulus in the WT and the Y8G/D9G α₇ structural ensembles. This was calculated by filling the heptagonal plane that comprises the annulus (see Fig. 1B), formed by G128 Cα (side length a= 7.52 ± 0.06 Å) at random with approximately 6000 points. Subsequently points were removed if they are closer than the van der Waals distance + 1.4 Å (where 1.4Å corresponds to the
radius of a water molecule) to any of the atoms in the structure to produce the accessible surface. Surface areas were calculated from the relation:

\[
\frac{7}{4}a^2 \cot \frac{\pi}{7} \frac{\text{Points}_{\text{remaining}}}{\text{Points}_{\text{total}}}
\]  

where \( \frac{7}{4}a^2 \cot \frac{\pi}{7} \) is the surface area of a heptagon with each side of length \( a \). The precision of the method due to its Monte Carlo character and the approximation of the \( \alpha \)-annulus as a heptagonal plane is ±4 Å².

**Proteolysis assays**

Proteolysis assays were performed using a SpectraMax M5 96-well microplate reader at 45 °C, in a buffer of 50 mM potassium phosphate, pH 7.5, 100 mM NaCl. Cleavage of 10 µM of a 9 residue peptide, Mca-RPPGFSAFK(Dnp)-OH (R&D Systems, MN, USA) (Mca: (7-Methoxycoumarin-4-yl)acetyl; Dnp: 2, 4-Dinitrophenyl), was followed for 45 min at \( \lambda_{\text{Ex}} = 320 \text{ nm} \) and \( \lambda_{\text{Em}} = 405 \text{ nm} \) in a total volume of 120 µL (after a 15 minute equilibration period). Below the N-termini of the constructs used in the assays are listed:

<table>
<thead>
<tr>
<th>( \alpha ) construct</th>
<th>1</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>GAMGMQQGAMAYDRAITV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y8G/D9G</td>
<td>GAMGMQQGAMAGGRAITV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \Delta(-3–4) )</td>
<td>GSAYDRAITV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \Delta(-3–11) )</td>
<td>GTV</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The first four residues (GAMG) are due to a cloning artifact that has been retained in constructs because M-1 gives rise to intense correlations in NMR spectra that are used as probes. These additional residues had no affect on spectra (i.e. 3 peaks were observed for M1 of Δ(-3–1) α,α, with intensity ratios of ‘in’ (B+C) and ‘out’ that were the same as for WT) or proteolysis rates (no differences were observed in proteolysis rates between WT and Δ(-3–4), see Fig. 4A).
**Supplementary Figures**

**Fig. S1. Pervasive µs-ms time-scale dynamics in α7.**

Ribbon diagram showing a single α-subunit highlighting those regions of structure for which backbone amide $^1$H-$^{15}$N correlation peaks were not observed in $^{15}$N-TROSY based spectra of α7 (indicated in red) (S2). Also shown is the α-annulus that is composed of residues from all 7 α-subunits. A large portion of the ‘invisible’ residues are localized in and around the gating area, including from M1 to S35. The absence of resonances provides strong evidence of conformational exchange.
Fig. S2. Ile probes in the gating region give rise to weak cross-peaks in $^1$H-$^{13}$C HMQC spectra.

Ile probes were initially placed into the gating termini of the α-subunits via mutagenesis in order to probe structure and dynamics in this region of the proteasome. Ile residues were inserted at positions 1, 4 and 7. Shown are the $^1$H-$^{13}$C HMQC datasets recorded on U-$^2$H, Ile-$[^{13}$CH$_3$δ1] α$_7$, 50°C, 600 MHz. Although strong peaks are observed for the majority of Ile δ1 methyl groups, only very weak correlations are obtained for I1, 4 or 7 (red), very likely reflecting the same µs-ms time-scale exchange process that degrades the $^1$H-$^{15}$N spectrum of α$_7$. Ile are therefore not particularly good probes of the gate structure and dynamics, but Met residues are much better (see text).
Fig. S3. Assignment of Met $^1$H-$^{13}$C HMQC correlations.

Assignments of methyl $^1$H and $^{13}$C chemical shifts of Met residues have been obtained via a mutation strategy in which the Met in question is mutated to Ala or Ile (a,c,e,f). Alternatively, in some cases all Met residues save one are mutated so that only correlations from the remaining Met methyl group are observed (b,d). Spectra are recorded on the WT $\alpha_7$ (a-f) or Y8G/D9G $\alpha_7$ (g), 50°C, 600 MHz. In each case the ‘black’ spectrum is that obtained for the mutant (where one or more Met are replaced), with the ‘red’ single contours indicating the position of peaks in a spectrum recorded with no Met mutations. Note that the effect of mutation is often to shift the position of cross-peaks slightly. For example, in the case of the ‘M-1 only $\alpha_7$ spectrum’ (Fig. S3b) B and C correlations are degenerate so that only a single peak for the ‘minor states’ is observed at $(\omega_C, \omega_H) = (17.1 \text{ ppm}, 1.78 \text{ ppm})$, with a relative intensity (with respect to the A state peak) that is expected for the sum of states B and C. A weak correlation from Met 120 was also assigned via mutagenesis, at $(\omega_c, \omega_h) = (19.3 \text{ ppm}, 1.86 \text{ ppm})$ (not shown).
Fig. S4. Cross-peaks from states A, B and C are also observed in Met $^1$H-$^{13}$C HMQC spectra of an $\alpha_7\alpha_7$ construct starting at G0.

The $^1$H-$^{13}$C Met HMQC spectrum of $\alpha_7\alpha_7$ (50°C, 600 MHz) where the first 3 residues have been truncated, $\Delta(-3\rightarrow1)$, shows that additional correlations are observed for M1, much like what is seen in spectra recorded on the ‘full length’ construct (with sequence shown in Fig. 1C). This establishes that the B,C cross-peaks are not an artifact of the additional residues at the N-terminus in the constructs that we have used. Notably, the relative intensities of the cross-peaks are: A 78±2%, (B+C)=22±2%, very similar to what is measured for both WT $\alpha_7$ and WT $\alpha_7\beta_7\beta_7\alpha_7$. 


Fig. S5. Paramagnetic relaxation enhancements.

A paramagnet is attached to Y8G/D9G α7 at positions 4 (a), 95 (b) or 107 (c) (1 at a time) by mutating G4, S95 or V107 to Cys and adding spin-label (see Materials and Methods). 1H $R_2$ values are measured on both oxidized (red) and reduced (black) samples by recording a series of two-dimensional data sets as a function of a parametrically varied transverse relaxation delay, $T$. Fitted peak heights, $I$, are converted to $R_2$ rates according
to the relation \( I = I_0 \exp(-R_2T) \). Shown are the decay profiles for selected \(^1\text{H}^N\) nuclei (a) and \(^1\text{H}\) methyl spins (b,c), as well as PRE rates, \( \Gamma_2 = R_2^{\text{at}} - R_2^{\text{red}} \). Errors are estimated by the covariance matrix method (S7). Similar measurements have also been performed on WT \( \alpha_7 \) samples. Measurements are recorded at 50°C, 600 MHz.
Fig. S6. Assignment of correlations from ‘in’ and ‘out’ states for WT α₇.

Superposition of $^1$H-$^{13}$C Met-methyl HMQC spectra of the WT α₇ ring showing that attachment of a spin-label at position 95 eliminates correlations from states B and C for M1, -1 (top), while a nitrooxide positioned at residue 20 eliminates peaks from the A conformation (bottom). Shown are spectra recorded with the nitrooxide in the oxidized (black) and reduced states (red; 1 contour). Identical results are obtained for Y8G/D9G α₇ as illustrated in Fig. 2B.
Fig. S7. Comparison of $^1$H$^N$ PRE rates measured for Y8G/D9G $\alpha_7$, and WT $\alpha_7$.

Correlation plot of $\Gamma_2^{NH}$ values measured for Y8G/D9G $\alpha_7$ (Y-axis) vs. WT $\alpha_7$ (X-axis) with spin-label attached to position 4 in both cases shows that many of the residues in the double mutant have increased rates. These residues are localized to helices 1 and 2, in particular, that are well inside the lumen of the ring. The results are consistent with the fact that on average 3 of the 7 N-termini of Y8G/D97 $\alpha_7$ are localized to the ‘inside’ of the lumen, while only 2 of the 7 are ‘in’ for WT $\alpha_7$ (see text).
Fig. S8. Placement of a spin-label within the lumen of the 11S activator eliminates the A (‘out’) state correlations in spectra of the α7-11S complex.

Space filling cross-section side-view representation of the α7-11S complex (pdbId: 1YA7 (S11)), highlighting the position of the spin-label in the lumen of 11S (at position 108 in each of the seven copies of the symmetric heptamer, yellow). The termini in the ‘out’ positions can potentially get ‘close’ to the spin-label, while those extending into the α7 cavity, corresponding to the antechamber in the intact proteasome, are distal (> 29 Å) from the nitrooxide. ¹H-¹³C HMQC spectra of the Y8G/D9G α7-11S complex with spin-label in the oxidized and reduced states are shown in black and red (single contour), respectively. The absence of A state correlations for both M1, -1 and 6 provides very strong evidence in support of the assignments of A and B/C peaks to the ‘out’ and ‘in’ conformations, respectively, cross-validating the results of other experiments presented in this work. Additional support of our model in which a subset of the termini are localized ‘out’ and ‘in’ the lumen of the barrel is provided by chemical shift data recorded on α5α7 showing that deletion of the gating residues results in significant changes in chemical shifts of methyl group probes located up to 28 Å from G128 of the α-annulus, well within
the antechamber ($\mathcal{S}I$), only possible in the case where some of the termini reside well within the barrel structure.
Fig. S9. The E25P mutation that destabilizes H0 opens the proteasome gate.

Helix H0 is the first stable secondary structural element in the 20S CP and plays an important role in positioning the gating residues close to the pore (S10) (Fig. 1B). We postulated that disruption of this helix would eliminate the ‘in’ conformation because the termini would no longer be situated properly to penetrate the annulus. Insertion of a Pro in the middle of an α-helix is known to destabilize it by over 3 Kcal/mol (S17), effectively disrupting the helix. The $^1$H-$^{13}$C HMQC spectrum of E25P $\alpha_7$ (50°C, 600 MHz) shows that peaks from states B and C, reporting on termini inserted into the lumen of the proteasome barrel, are eliminated (black). For comparison, the corresponding spectrum of WT $\alpha_7$ is shown (red, single contour). This provides strong evidence of the importance of helix H0 for positioning the gating residues properly for insertion into the barrel. Gly substitutions have similar effects as Pro but are not as pronounced (helix destabilization by almost 1 Kcal/mol (S17)) and the population of the ‘out’ state is increased from approximately 75% to approximately 90% (fig. 2C).
Fig. S10. 11S binds Y8G/D9G α7.

(a) Met region of a $^1$H-$^{13}$C HMQC spectrum (600 MHz, 40°C) of a Y8G/D9G α7-11S complex where Y8G/D9G α7 is labeled as U-$^2$H,$^1$H-Met-$[^{13}$CH$_3]$,-Ile-$[^{13}$CH$_3]$,-Leu,-Val-$[^{13}$CH$_3$, $^{12}$CD$_3$] (S18) and 11S is unlabeled (red). Shown also is the corresponding spectrum of Y8G/D9G α7 in the absence of 11S (black). The addition of excess WT 11S to Y8G/D9G α7 results in significant changes to the Met chemical shifts of the major state (A), that reports on the ‘out’ conformation of the gate, with little effect on the shifts of the peaks reporting on the minor states (B,C). This provides direct evidence that 11S
binds Y8G/D9G specifically, consistent with the PRE results of Figure S8 where addition of 11S with a spin label at position 108 to U-\(^2\)H,\(^1\)H-Met-\([\varepsilon^{13}\text{CH}_3]\)-Y8G/D9G \(\alpha_7\) leads to the elimination of the Met peaks derived from the out conformation.

(b) Ile region of the same spectrum recorded in (a) showing further evidence of binding of 11S to Y8G/D9G \(\alpha_7\). The results in Figure S8 and the present figure establish that 11S binds to the double Gly mutant of \(\alpha_7\) and that the lack of 11S activation of Y8G/D9G \(\alpha_7\) (i.e., lack of change in relative populations of ‘in’/’out’ states) is not due to absence of binding. Rather 11S is unable to stabilize the ‘out’ conformation of the Y8G/D9G \(\alpha_7\), as has been reported previously in the context of the full proteasome (S11, S19).
Fig. S11. An 11S-PAN chimera increases the rates of proteolysis of WT proteasome.

A chimeric construct was constructed, where the last 10 residues of WT 11S were replaced with the sequence EPAHLDVLYR, derived from PAN’s extreme C-terminus (S20). Addition of this 11S chimera to the Thermoplasma acidophilum proteasome (4 nM, monomer concentration) resulted in increased proteolysis rates for the WT protein (black traces) but not for the Y8G/D9G gating mutant (blue traces; same concentration range of 11S was used as for WT), consistent with expectations from recently published crystal structures of the chimera proteasome complex (S21, S22). Essentially no change in the populations of the ‘in’/’out’ states of the gate were quantified upon binding excess of the chimera to the Y8G/D9G proteasome mutant (‘in’ = 45, ‘out’ = 55 for apo Y8G/D9G α7; ‘in’ = 47, ‘out’ = 53 for Y8G/D9G α7 bound to the chimera; error ±2%). In contrast, much larger changes were observed for binding to WT α7 (‘in’ = 24, ‘out’ = 76 for apo WT α7; ‘in’ = 10, ‘out’ = 90 for WT α7 bound to the chimera; error ±2%).

Crystal structures of the 11S-PAN chimera proteasome complex (S21, S22) establish that it is the interactions with the PAN peptide portion of the activator that are
driving gate opening. Thus, our results (this figure and Figure 2C), establish that the mechanism of action of both 11S and PAN binding to the WT proteasome is to shift the relative populations of ‘out’ and ‘in’ so as to decrease the number of ‘in’ termini and hence open the proteasome gate.
## Supplementary Tables

**Table S1. Summary of structural restraints and statistics for 30 (50) lowest energy structures for residues -3–13 of WT (Y8G/D9G) α7.**

<table>
<thead>
<tr>
<th>Construct</th>
<th>WT α7(2ku1)</th>
<th>Y8G/D9G α7(2ku2)</th>
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<tbody>
<tr>
<td><strong>A. Structural restraints</strong></td>
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<tr>
<td>Methyl PREs</td>
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<td></td>
</tr>
<tr>
<td>‘in’</td>
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<tr>
<td>‘out’</td>
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<tr>
<td>Amide PREs</td>
<td>19</td>
<td>26</td>
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<tr>
<td><strong>B. Statistics for the accepted structures</strong></td>
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<tr>
<td>E(overall)</td>
<td>53 ± 38</td>
<td>130 ± 37</td>
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<tr>
<td>E(angle)</td>
<td>429 ± 13</td>
<td>414 ± 14</td>
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<tr>
<td>E(bond)</td>
<td>120 ± 2</td>
<td>116 ± 3</td>
</tr>
<tr>
<td>E(improper)</td>
<td>198 ± 15</td>
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</tr>
<tr>
<td>E(rama)</td>
<td>-904 ± 36</td>
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<tr>
<td>E(van der Waals)</td>
<td>211 ± 5</td>
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<tr>
<td>E(noel)</td>
<td>0.1 ± 0.2</td>
<td>0.1 ± 0.1</td>
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<tr>
<td><strong>C. Ramachandran analysis (%)</strong></td>
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<tr>
<td>Residues in most favoured regions</td>
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<td>Residues in additional allowed regions</td>
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<td>Residues in generously allowed regions</td>
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<tr>
<td>Residues in disallowed regions</td>
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</tr>
</tbody>
</table>

*Statistics are based on 10 structures for each of the 3 (5) permutations of arranging 2 (3) N-termini ‘in’ for WT (Y8G/D9G). Thus, the statistics are averages over 30 WT (50 Y8G/D9G) structures.*
References